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再生医療実用化研究事業

間葉系幹細胞を用いた移植治療における品質及び安全性判定基準の確立に関する研究

平成20年度　総括研究報告書

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厚生労働科学研究費補助金（再生医療実用化研究事業）
総括研究報告書

間葉系幹細胞を用いた移植治療における品質及び安全性判定基準の確立に関する研究

研究代表者 青山 朋樹　京都大学再生医科学研究所

研究要旨
間葉系幹細胞はさまざまな組織に分化可能な組織幹細胞である。このため疾患、外傷などで傷害を受けた組織の修復・再生に期待され、既に臨床応用も開始されている。しかしながら間葉系幹細胞の定義が曖昧であることや施設、技術者によって抽出の方法が異なるなどの理由からその品質を保証する品質管理基準は作成されていない。また*In Vitro*の体外培養において自然発癌例が報告されるなど安全性の監視機構の構築の必要性も高まってきている。
本研究においては品質管理及び安全性監視機構を構築し、臨床試験「難治性骨壊死に対するMSC細胞移植治療」においてその妥当性を検討する。
3年間の間に研究結果を明らかにでき、患者、社会、医療従事者、行政にその情報を還元できる事を特徴としている。

A.研究目的

本研究の目的は間葉系幹細胞(Mesenchymal stem cell, MSC)を用いた細胞医療を行う際の培養（行程あるいは課程）における品質評価及び安全性監視機構を構築することである。

B.研究方法

本研究は平成19年度に京都大学整形外科で開始された臨床試験「難治性骨壊死に対するMSC細胞移植治療」と連携して実施する。品質評価項目はMSCの①多分化能評価②増殖能評価であり、安全性監視項目は③感染の監視④癌化の監視である。本研究においては*In Vitro*でこれらの4項目を定量的評価し、基準値を作成し、そのプロトコールを臨床試験にフィードバックする。最終的には臨床試験で得られた問題点を検討し、安全で品質の高い細胞移植治療プロトコールを作成する。

（倫理面への配慮）解析方法の検討に用いるドナー由来のMSCは、京都大学医学部医の倫理委員会の審査を経て実施した。臨床試験は「ヒト幹細胞を用いる臨床研究に関する指針」を遵守しインフォームドコンセントを得られた症例において個人情報の保護・秘匿を遵守して行う予定である。

C.研究結果

分化能の確認は*In Vitro*で分化誘導を行い、特異的基質産生を定量評価する方法は後追いの検査結果報告となるためCD106などの分化特異的細胞表面マーカーの有用性を検討している。増殖能予測の指標として増殖因子PDGFおよびTGFb1の測定の有用性が明らかになった。感染監視は cGMPグレードに準拠したプロトコール、SOP、定期検査方法を確立し、有効性を検証している。癌化監視は早期悪性転化予測マーカーとしてp16遺伝子転写調節領域のメチル化解析の有効性を明らかにし、染色体解析、免疫不全マウスへの皮下接種法と比較したが後2者の結果判定は移植後の後追い報告となり実用的でないことが明らかになった。有用性については腫瘍形成が認められないため、いずれの検査でも判定困難である。

D.考察およびE.結論

平成18、19年度に作成した品質評価、安全性監視のためのプロトコールを用いて平成19年度より臨床試験「難治性骨壊死に対するMSC細胞移植治療」を実施した。臨床試験を完遂し、プロトコールの問題点の検討、妥当性を検証していく予定である。

F.健康危険情報

該当なし

G.研究発表

1.論文発表

1) Otsuka S, Aoyama T, et al. PGE2 signal via EP2 receptors evoked by a selective agonist enhances regeneration of injured articular cartilage. Osteoarthritis Cartilage. 2009 17(4):529-38.

2.学会発表

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H.知的財産権の出願・登録状況

「生体由来物用搬送装置」特願2007-194906、現在PCT出願中

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
青山朋樹、戸口田淳也	間葉系幹細胞	中辻憲夫	幹細胞の分化誘導と応用	エヌ・ティー・エス	東京	2009	55-61
青山朋樹、戸口田淳也 中村孝志	間葉系幹細胞を利用した大腿骨頭無腐性壊死の再生医療技術	中辻憲夫	幹細胞の分化誘導と応用	エヌ・ティー・エス	東京	2009	262-7

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Osteoarthritis and Cartilage

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PGE2 signal *via* EP2 receptors evoked by a selective agonist enhances regeneration of injured articular cartilage

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Summary

Objective: The effect of the prostaglandin E2 (PGE2) signal through prostaglandin E receptor 2 (EP2) receptors on the repair of injured articular cartilage was investigated using a selective agonist for EP2.

Methods: Chondral and osteochondral defects were prepared on the rabbit femoral concave in both knee joints, and gelatin containing poly-lactic-co-glycolic acid microspheres conjugated with or without the EP2 agonist was placed nearby. Animals were sacrificed at 4 or 12 weeks post-operation, and regenerated cartilage tissues and subchondral structure remodeling were evaluated by histological scoring. The quality of regenerated tissues was also evaluated by the immunohistochemical staining of EP2, type II collagen, and proliferating cell nuclear antigen (PCNA). As an evaluation of side effects, the inflammatory reaction of the synovial membrane was analyzed based on histology and the mRNA expression of matrix metalloproteinase3 (MMP3), tissue inhibitor of metalloproteinase 3 (TIMP3), and interleukin-1β (IL-1β). Also, the activity of MMP3 and the amount of tumor necrosis factor-α (TNF-α) and C-reactive protein in joint fluid were measured.

Results: In both models, the EP2 agonist enhanced the regeneration of the type II collagen-positive tissues containing EP2- and PCNA-positive chondrocytes, and the histological scale of regenerated tissue and subchondral bone was better than that of on the control side, particularly at 12 weeks post-operation. No inflammatory reaction in the synovial membrane was observed, and no induction of pro-inflammatory cytokines was found in joint fluid.

Conclusion: Selective stimulation of the PGE2 signal through EP2 receptors by a specific agonist promoted regeneration of cartilage tissues with a physiological osteochondral boundary, suggesting the potential usefulness of this small molecule for the treatment of injured articular cartilages.
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Key words: PGE2, EP2, Agonist, Cartilage, Defect, Repair, Therapeutic drug, Osteoarthritis.

Introduction

Chondrocytes in articular cartilage are differentiated cells with minimum proliferating potential and low metabolic activity[1]. Because these cells are fully responsible for the production of cartilage matrix consisting of collagens and proteoglycans, considerable damage to articular cartilage is unrepairable, initiating a sequence of catabolic events leading to a pathological condition known as osteoarthritis (OA). Although inflammation of the synovium and the destruction of subchondral bone integrity also play an important role in the progression of OA, the poor regenerative capacity of chondrocytes is the major disease-causing factor[1,2]. In the early stages of OA, however, not only catabolic but also anabolic activity is enhanced in chondrocytes. As catabolic activity, chondrocytes produce several catabolic cytokines such as interleukin-1 (IL-1), which in turn induce the production of proteinases such as matrix metalloproteinases (MMPs) and a disintegrin-like and metalloprotease with thrombospondin (ADAMTS) leading to the destruction of the matrix network[2]. As anabolic activity, chondrocytes produce anabolic cytokines such as the bone morphogenic protein (BMP) family and insulin like growth factor-1 (IGF-1), which induce the synthesis of collagens and initiate the proliferation of chondrocytes themselves making osteophytes at the periphery[2]. A disruption of the equilibrium between the catabolic and anabolic activities results in catastrophic damage to articular cartilage. In adult articular cartilage, the equilibrium leans toward catabolic activity; the proliferation of chondrocytes is decreased and the subchondral structure is weak.

The role of prostaglandin E2 (PGE2) in the development of OA is controversial. Pro-inflammatory signal mediators such as IL-1 and tumor necrosis factor-α (TNF-α) induce

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the synthesis of PGE2 by promoting the expression or activities of cycloxygenase (COX)-2 and microsomal PGE synthase-1[3]. The synthesized PGE2 promotes IL-1 expression as a positive feedback mechanism, degrades the cartilage matrix[4], and finally induces apoptosis of chondrocytes[5], indicating a catabolic role for PGE2 in OA. In some reports, however, anabolic effects of PGE2 were demonstrated[1,3]. PGE2 opposed the effect of IL-1 by down-regulating type I collagen[6] and stimulating type II collagen gene expression[7,8]. Also, PGE2 stimulated the synthesis of proteoglycan and collagen through the induction of IGF-1-binding protein[9], and induced the proliferation of rat chondrocytes as demonstrated by an increase in the incorporation of [^{3}H]-thymidine[9].

Several factors are involved in these controversial findings including experimental design, the level of PGE2 expression, the balance with other pro-inflammatory cytokines, and most notably, the variety of receptors. PGE2 exerts its biological effect through one of four receptors, EP1, EP2, EP3, or EP4. The development of specific agonists and antagonist for each receptor enables the understanding of the receptor-specific signal transduction mechanism and its consequence. Signals through EP1 and EP3 coupled by G_i protein increase the intra-cellular Ca^{2+} concentration, and those through EP2 and EP4 coupled by G_s protein increase cyclic adenosine monophosphate (cAMP). Although the second messenger is common, the amide acid identity is only 31% between EP2 and EP4. EP4 (513 amino acids) has the longest intra-cellular C terminal, but EP2 (362 amino acids) has a compact structure. In osteoclastogenesis, the EP2 and EP4 mediate the induction of receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL), but the extent of the contribution by each receptor is different. Although both EP2 and EP4 are expressed in dendritic cell, EP4 has selective action for cell migration. This selectivity of EP4 may be caused by the fact that EP4 but not EP2 couples to phosophatidylinositol 3-kinase in addition to cAMP[10].

We have previously demonstrated that EP2 was the major PGE2 receptor in articular chondrocytes[11]. And a specific agonist for EP2 not EP4 promoted the growth of mouse and human chondrocytes by up-regulating the expression of growth-promoting genes such as the *cyclin D* gene stimulating the increase of cAMP[11]. The protein structure and function of EP2 are conserved between many species, and the amino acid homology between the human and mouse, rat, or rabbit is 88.2, 84.9, and 90.2%, respectively. The effect of the EP2 agonist on chondrocytes was confirmed in a rat organ culture system, suggesting the possible application of this small molecule as a new therapeutic tool for injured articular cartilage[11].

In this study, we investigated the effect of an EP2 agonist on injured articular cartilage *in vivo* using rabbit knee joints and also on other joint structures such as the synovium and subchondral bone.

Materials and methods

REAGENTS

Microspheres loaded with the selective EP2 agonist, ONO-8815Ly[12] prepared by the emulsion–solvent evaporation method as described[13]. Briefly, ONO-8815Ly was dissolved in purified water as the inner water phase and polylactic-co-glycolic acid (PLGA) was dissolved in dichloromethane as the oil phase. The water/oil (w/o) emulsion was gradually added to the outer water phase containing polyvinyl alcohol (PVA, 0.1%, w/v), NaCl (2%, w/v) and maltose (2%, w/v) adjusted to pH 3.0, under stirring with a turbine-shaped mixer at 5000 rpm to obtain a water/oil/water emulsion. Then PLGA microspheres were formed in the outer water phase after the evaporation of dichloromethane. In order to recover the microspheres without a free form of ONO-8815Ly, the suspension was centrifuged at 3000 rpm for 10 min and the microspheres were precipitated. The washed microsphere precipital was lyophilized to remove residual organic solvent and water, and then dried solid ONO-8815Ly-loaded microspheres were recovered. ONO-8815Ly-loaded microspheres were dispersed in purified water, and then gelatin aqueous solution (20%, w/w) was poured into the microsphere suspension. The resultant microsphere–gelatin suspension was poured into a polypropylene container and placed in a refrigerator for 12 h to form a gel. Afterward, glutaraldehyde aqueous solution (12.5 μg/ml) was poured into the microsphere–gelatin gel and placed in the refrigerator for 24 h for the crosslink reaction. The gel sheet obtained was placed into glycine aqueous solution. These procedures were performed repeatedly. Small cylinder-shaped gelatin hydrogels containing either 80 or 400 μg of ONO-8815 were obtained by hollowing out the gelatin hydrogel sheet.

SURGICAL PROCEDURE

The institutional animal research committee, according to the guidelines for Animal Experiments of Kyoto University, approved this investigation. Japanese white rabbits (Shimizu Laboratory Supplies Co., Kyoto, Japan) were at least 5 months old and had a body weight of 3 kg.

Two types of cartilage defect on the femoral concave of the patello-femoral joint were made according to depth (Supplementary Fig. 1). A chondral defect, which involved the osteochondral boundary (tide mark) but not subchondral bone, was made using a punch without damaging the subchondral bone (5.0 mm in diameter) [Supplementary Fig. 1(A–C)]. An osteochondral defect was made using a hand drill [4.0 mm in diameter and 5.0 mm in depth, Supplementary Fig. 1(D–F)]. By preliminary experiments, we have confirmed that both types of defects were not healed spontaneously (data not shown).

The same type of defect was created in both femurs, and a cylinder-shaped gelatin hydrogel containing PLGA microspheres conjugated with ONO-8815 (80 or 400 μg) was placed into the infra-patellar fat pad on one side (thereafter designated EP2 agonist-treated samples), and gelatin hydrogels without microspheres were placed on the contralateral side (contralateral samples). The animals were allowed to move. To exclude any possible effect of ONO-8815 in the systemic circulation, controls were established, in which empty gelatin hydrogel was placed in bilateral knee joints after creating each defect model (control samples, $N=3$). Animals were sacrificed and evaluated at 4 ($N=5$) and 12 ($N=8$) weeks after the operation.

HISTOLOGICAL EVALUATION

Cartilage samples were fixed overnight at 4°C in a 10% formalin solution, decalcified by formic acid, and embedded in paraffin. Then sections were cut at 6 mm through the center of each defect, stained with Safranin-O/Fast Green and hematoxylin–eosin (HE), examined in a blinded manner by two evaluators, and graded with the use of a modified Wakitani histological scale[14]. The reconstitution of articular chondrocytes and subchondral bone connections (category II on the modified Wakitani scale) was partially evaluated by grading from 0 to 7. Specimens of the synovium around the infrapatellar fat pads were fixed in 10% formalin, embedded in paraffin, and cut into 6 μm thick sections for histological evaluation. Sections were stained with HE and the severity of synovial lesions was graded according to the histological scoring system, based on the hyperplasia of synovial lining cells, hypertrophy of the synovial lining layer, infiltration of inflammatory cells, proliferation of granulation tissue, and vascularization[15]. Two independent observers blinded to the treatment groups graded all sections.

IMMUNOHISTOCHEMISTRY

Immunostaining of proliferating cell nuclear antigen (PCNA; diluted 1:100; Dako, Glostrup, Denmark) and alpha1 type II collagen (diluted 1:50; Daiichi Fine Chemical, Toyama, Japan) was performed as previously mentioned[11]. Immunostaining of EP2 (diluted 1:100; Cayman Chemical Co., Ann Arbor, MI) was performed as described by Fukuda *et al.*[16]. Under the microscope (400×), all cells and PCNA-positive cells were counted by two individuals. Three visual fields were randomly selected by each observer, and, therefore, each specimen was evaluated three times. Then labeling index was calculated as the mean of these three values. The relative increase in PCNA-positive cells was expressed as the ratio of the labeling index of the EP2 agonist-treated sample vs that of the contralateral sample. The labeling index of control sample was also calculated as a value relative to those of contralateral sample.

ASSAY OF MMP3, TNF-α, AND C-REACTIVE PROTEIN (CRP) IN SYNOVIAL FLUID

After the injection of 1 ml of saline solution into the knee joint, synovial fluid was obtained by arthrocentesis through a sub-patellar approach. Synovial fluid was aspirated as far as possible from the knee joint. After

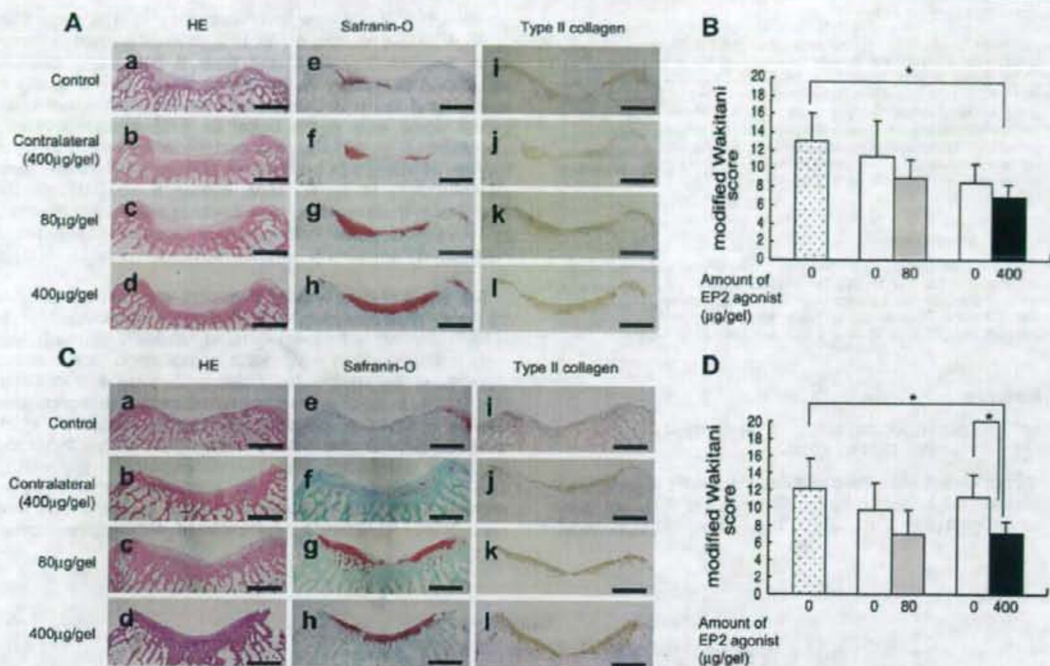

Fig. 1. Effects of EP2 agonist on the regeneration of chondral defects. Histological evaluations were performed at 4 (A) or 12 (C) weeks after the operation. Each specimen was prepared from control samples (a, e, and i), contralateral (b, f, and j), or EP2 agonist-treated (c, g, and k, 80 μg/gel; d, h and l; 400 μg/gel) samples. The quality of regenerated tissues was analyzed by HE staining (a–d), Safranin-O staining (e–h), or immunohistochemical staining with anti-type II collagen Ab (i–l). Magnification 2×. Bar = 2.0 mm. The quality of regenerated tissues was also evaluated with a modified Wakitani histological scale at 4 (B) and 12 (D) weeks after the operation. Dotted and open boxes indicate the scale for control and contralateral samples, respectively, $^{*}P < 0.05$.

the specimen was centrifuged at 1500 rpm for 20 min, the supernatant was drawn out and stored at −70°C[17]. MMP3 activity was measured with a MMP3 fluorimetric drug discovery kit (BIOMOL International LP, Plymouth Meeting, PA). TNF-α levels in joint fluids were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with specific anti-TNF-α polyclonal antibodies (Abs) (BD Pharmingen, San Diego, CA). Briefly, microtiter plates were coated with 50 μl of anti-rabbit. TNF-α capture Abs (4 mg/ml) overnight at 4°C, then washed twice with phosphate buffered saline (PBS) containing 0.05% Tween 20 and blocked overnight at 4°C with 10% fetal bovine serum (FBS) in PBS. After the plates were washed four times, standards and samples (100 μl) were incubated in duplicate overnight at 4°C. Plates were again washed and a biotin-conjugated anti-TNF-α secondary Ab (2 mg/ml) was added for 1 h at room temperature. A 30-min incubation with a 1:400 dilution of avidin–peroxidase (Sigma) followed extensive washing of the plate. Finally, TMBlue (Intergen, Milford, MA) substrate was added (100 μl/well) and incubated at room temperature for 30 min. The absorbance was read at 450 nm with an UVmax microplate reader (Molecular Devices, Menlo Park, CA). Rabbit CRP was measured with a rabbit CRP ELISA kit (Alpha Diagnostic International Inc., San Antonio, TX).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted from frozen synovial samples using TRIzol reagent (Invitrogen, Carlsbad, CA) and 1 μg was reverse transcribed for single-stranded cDNA using the oligo (dT) primer and Superscript II reverse transcriptase (Invitrogen). RT-PCR was performed in duplicate for each sample using primers listed in Table I[18,19].

Table I
Primers used in the RT-PCR analyses

Gene	Primers*	Size (bp)	Position	Accession no.
MMP3	CTGGAGGTTTGATGAGAAGA CAGTTCATGCTCGAGATTCC	336	1278–1297 1597–1616	NM_001082280
TIMP1	GCAACTCCGACCTTGTCATC AGCGTAGGTCTTGGTGAAGC	326	122–141 428–447	NM_001082232
IL-1β	TGCTGTCCAGACGAGGGCAT ACTCTCCAGCTGCAGGGTAG	473	210–229 664–683	NM_001082201
GAPDH	GTCAAGGCTGAGAACGGGAA GCTTCACCACCTTCTTGATG	613	246–265 839–858	NM_001082253

*All primer sequences are written from 5′ to 3′. The top sequence is the sense primer and the bottom sequence is the anti-sense primer.

QUANTITATIVE RT-PCR

The levels of mRNA expression of genes (*MMP3, TIMP1, IL-1β* and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*)) were quantified by SYBR Green (Applied Biosystems, Foster City, CA) real time PCR with the ABI PRISM 377 Sequence Detection System (Applied Biosystems). Each gene amplification efficiency was similar with *GAPDH*. All reactions were run in triplicate, and the mean value was used to calculate the ratio of target gene/*GAPDH* expression in each sample. Using the ratio in untreated sample as a standard (1.0), the relative ratio of the treated sample was presented as the relative expression level of the target gene[11].

STATISTICAL ANALYSES

All statistical analyses were performed using Statcel software. The results are shown as the mean ± SD. The analysis of variance (ANOVA) test was used to compare the differences in the scales between multiple groups. The Student's *T* test was used to compare the differences in the scales between two groups. A *P* value < 0.05 was considered significant.

Results

EP2 AGONIST PROMOTED TISSUE REGENERATION IN THE CHONDRAL DEFECT MODEL

Four weeks after the operation, the quality of regenerated tissues was analyzed by HE staining [Fig. 1(A, a–d)], Safranin-O staining [Fig. 1(A, e–h)], or immunohistochemical staining with anti-type II collagen Ab [Fig. 1(A, i–l)]. Most of regenerated tissues in EP2 agonist-treated samples were Safranin-O-positive and type II collagen-positive, suggesting the quality as hyaline cartilage. The quality of regenerated tissue evaluated by modified Wakitani histological scale was much better in EP2 agonist-treated samples than control samples, and the difference was statistically significant in the case of 400 μg/gel-treated samples [Fig. 1(B): 80 μg, $P = 0.08$; 400 μg, $P = 0.04$]. On the other hand, there were no statistically significant difference in histological scale between EP2 agonist-treated and contralateral samples [Fig. 1(B): 80 μg, $P = 0.21$; 400 μg, $P = 0.24$].

The effect of EP2 agonist treatment was much clear at 12 weeks after operation. In control and contralateral samples, the amount of regenerated tissues was much less than that found at 4 weeks after operation, and most of them were negative for Safranin-O or type II collagen [Fig. 1(C)]. In EP2 agonist-treated samples, regenerated tissues reached a considerable width, most of which were Safranin-O and type II collagen-positive indicating properties compatible with hyaline cartilage [Fig. 1(C)]. The histological scale of EP2 agonist-treated samples showed significantly better than that of control and also contralateral samples in the case of 400 μg/gel-treated

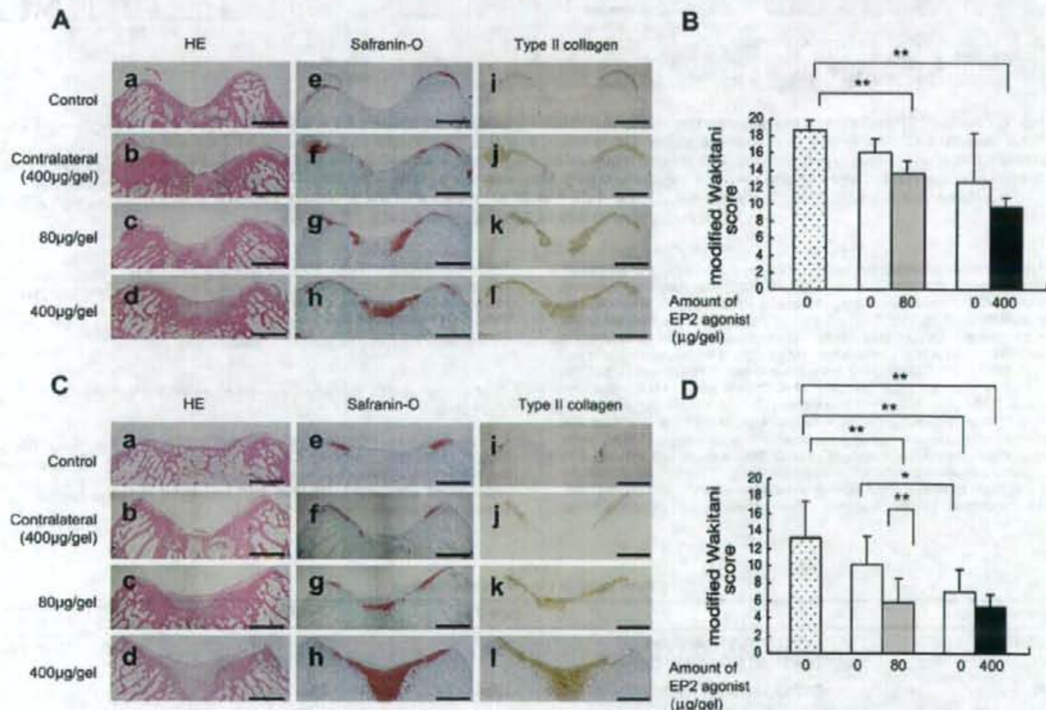

Fig. 2. Effects of EP2 agonist on the regeneration of osteochondral defects. Histological evaluations were performed at 4 (A) or 12 (C) weeks after the operation. Each specimen was prepared from control samples (a, e, and i), contralateral (b, f, and j), or EP2 agonist-treated (c, g, and k, 80 μg/gel; d, h and l; 400 μg/gel) samples. The quality of regenerated tissues was analyzed by HE staining (a–d), Safranin-O staining (e–h), or immunohistochemical staining with anti-type II collagen Ab (i–l). Magnification 2×. Bar = 2.0 mm. The quality of regenerated tissues was also evaluated with a modified Wakitani histological scale at 4 (B) and 12 (D) weeks after the operation. Dotted and open boxes indicate the scale for control and contralateral samples, respectively. $^{**}P < 0.01$; $^{*}P < 0.05$.

samples [Fig. 1(D): vs control, $P=0.02$; vs contralateral, $P=0.01$]. The histological scale of contralateral samples tended to be better than that of control samples, although there was no statistical significance [Fig. 1(C): 80 μg, $P=0.31$; 400 μg; $P=0.1$, Fig. 1(D): 80 μg, $P=0.2$; 400 μg; $P=0.35$].

EP2 AGONIST PROMOTED TISSUE REGENERATION IN THE OSTEOCHONDRAL DEFECT MODEL

To analyze the effect of the EP2 agonist on tissue regeneration in osteochondral lesions, an osteochondral defect model was created [Supplementary Fig. 1(C–E)]. At 4 weeks

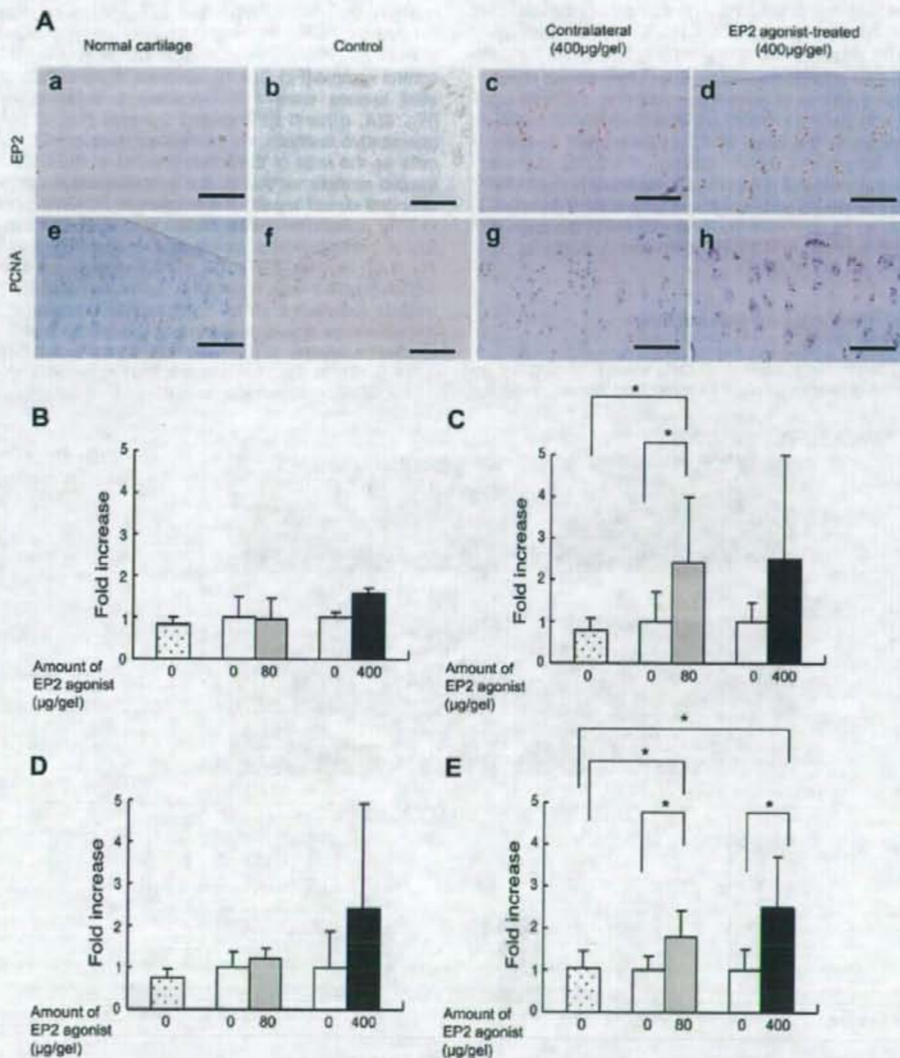

Fig. 3. EP2 agonist promotes proliferation of EP2-positive cells. A: Expression of EP2 and PCNA in normal and regenerated cartilage of chondral defect model. Specimens were prepared from a normal femur without any treatment (a and e), a control sample (b and f), a contralateral sample (c and g), and an EP2 agonist-treated (400 μg/gel) sample (d and h) at 12 weeks after the operation. Immunohistochemical staining was performed with anti-EP2 Ab (a–d) or anti-PCNA Ab (e–h). Bar = 100 μm. B–E: Quantitative analysis of PCNA-positive cells. Cells positive for PCNA staining were counted under the microscope, and a labeling index was calculated for each sample. The relative increase in PCNA-positive cells is expressed as the ratio of the labeling index of the EP2 agonist-treated sample (shaded bars) vs that of the contralateral sample (open bars). The labeling index of control sample relative to those of contralateral sample was indicated in dotted boxes. Samples were prepared from the chondral defect model at 4 weeks (B), chondral defect model at 12 weeks (C), osteochondral defect model at 4 weeks (D), and osteochondral defect model for 12 weeks (E). $^{*}P<0.05$.

after the operation, the osteochondral defects were filled with regenerated tissue in all cases, but there was a significant difference in histological scale between EP2 agonist-treated and control samples [Fig. 2(D): 80 μg, $P = 0.005$; 400 μg, $P = 0.0003$]. As in the chondral defect model, the difference was not significant between EP2 agonist-treated than contralateral samples [Fig. 2(D): 80 μg, $P = 0.07$; 400 μg, $P = 0.21$].

At 12 weeks after operation, the effect of EP2 agonist treatment was much evident. The osteochondral defect of EP2 agonist-treated samples was filled with Safranin-O and type II collagen-positive tissues [Fig. 2(C)], and the grading scale of EP2 agonist-treated samples showed and significantly better than that of control samples [Fig. 2(D): 80 μg, $P = 0.002$; 400 μg, $P = 0.0003$] and also than that of contralateral samples in the case of 80 μg/gel-treated samples [Fig. 2(D): 80 μg, $P = 0.006$; 400 μg, $P = 0.05$]. As we observed in the chondral defect model, the scale of contralateral samples tended to be better than that of control samples, and the difference was statistically significant in the case of contralateral sample in 400 μg/gel-treated animals at 12 weeks [Fig. 2(D), $P = 0.005$].

EP2 AGONIST STIMULATED THE PROLIFERATION OF EP2-POSITIVE CELLS

In normal cartilage, almost all cells were EP2-positive as we previously demonstrated in mice and human articular cartilage[11] [Fig. 3(A, a)]. Almost all cells in regenerated tissues of chondral defect model at 12 weeks after operation expressed the EP2 receptor in control [Fig. 3(A, b)], contralateral [Fig. 3(A, c)] and EP2-treated samples (400 μg/gel) [Fig. 3(A, d)]. Identical results were observed in samples harvested at 12 weeks after operation (data not shown), suggesting that the cartilage regeneration was conducted mainly by EP2-positive cells. To evaluate the proliferating ability of these EP2-positive cells, same specimens were used for PCNA staining [Fig. 3(A, e–h)]. Almost no cells were PCNA-positive in normal cartilage [Fig. 3(A, e)] and control sample [Fig. 3(A, f)], whereas some cells in regenerated tissues were PCNA-positive both in contralateral [Fig. 3(A, g)] and EP2-treated samples [Fig. 3(A, h)]. For quantitative analysis, we compared relative PCNA-positive cells as the ratio of the labeling index of the EP2 agonist-treated sample vs that of the contralateral sample. In the chondral defect model, the fraction of PCNA-positive cells in EP2 agonist-treated samples was almost the same as that in contralateral samples at 4 weeks [Fig. 3(B): 80 μg, $P = 0.47$; 400 μg, $P = 0.19$]. At 12 weeks, the fraction of PCNA-positive cells seemed to be higher in EP2 agonist-treated samples than in contralateral samples, although the difference was not convincing due to the wide variation among samples [Fig. 3(C): 80 μg, $P = 0.04$; 400 μg, $P = 0.12$]. In the osteochondral defect model, the fraction of PCNA-positive cells in EP2-treated samples was not

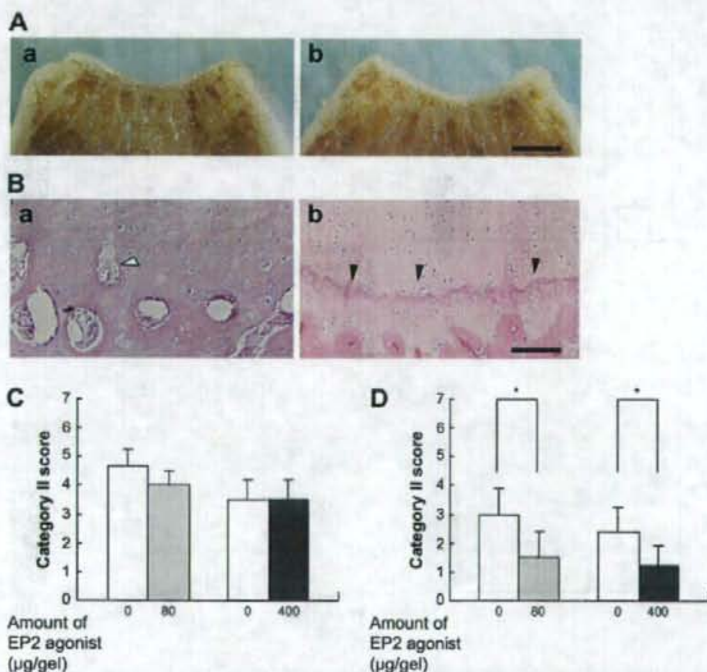

Fig. 4. Effect of EP2 agonist on remodeling of the deep layer zone in chondral defect model. A: Macroscopic view of specimens from contralateral sample (a) and EP2 agonist-treated (400 μg/gel) sample (b) of chondral defect model at 12 weeks after the operation. Bar = 4.0 mm. B: Microscopic view of the osteochondral boundary in the specimens presented in (A). (a) Contralateral sample, (b) EP2 agonist-treated (400 μg/gel) sample. Note that there was no clear boundary, and vascular invasion into articular chondrocytes (white arrowhead) was observed in (a), whereas a clear tidemark (black arrowhead) was formed in (b). Magnification 40×. Bar = 100 μm. C and D: Quantitative evaluation of the osteochondral boundary. The boundary formed between articular cartilage and subchondral bone was evaluated by category II scale of the modified Wakitani scale (0–7, 7 is worse). Samples were prepared from the chondral defect model at 4 weeks (C) and at 12 weeks (D). $^*P < 0.05$.

different from those in contralateral samples at 4 weeks [Fig. 3(D): 80 μg, $P=0.31$; 400 μg, $P=0.46$], but significantly higher at 12 weeks [Fig. 3(E): 80 μg, $P=0.02$; 400 μg, $P=0.02$]. In all settings, the labeling index of control samples showed no difference from those of contralateral samples [Fig. 3(B): $P=0.47$; Fig. 3(C): $P=0.31$; Fig. 3(D): $P=0.1$; Fig. 3(E): $P=0.43$].

EP2 AGONIST REPAIRED THE OSTEOCHONDRAL BOUNDARY

Reconstruction of the physiological boundary between articular cartilage and underlying bone tissue is important to maintain the mechanical and biological properties of articular cartilage. Macroscopical examination of EP2 agonist-treated (400 μg/gel) samples in chondral defect model at 12 weeks after operation showed a clear boundary between articular cartilage and subchondral bone [Fig. 4(A, b)]. Microscopical examination demonstrated the reconstruction of the tidemark [Fig. 4(B, b)]. These findings were not observed in contralateral samples. The boundary was not clear in macroscopical examination [Fig. 4(A, a)], and microscopical examination also showed no boundary with some vascular structures in the cartilaginous portion [Fig. 4(B, a)]. The difference was quantitatively evaluated using the category II scale of the modified Wakitani scale. There was no significant difference between EP2 agonist-treated and contralateral samples at 4 weeks [Fig. 4(C)], but the scale was significantly lower in the former than the latter at 12 weeks after operation [Fig. 4(D): 80 μg, $P=0.03$; 400 μg, $P=0.04$].

Similar findings were obtained in the osteochondral defect model (Fig. 5). EP2 agonist-treated (400 μg/gel) samples in osteochondral defect model at 12 weeks after operation the showed a clear boundary between articular cartilage and subchondral bone by macroscopical and microscopical examinations [Fig. 5(A, b and B, b)], which were not observed in contralateral samples [Fig. 5(A, a and B, a)]. Quantitative evaluation demonstrated that the boundary was much better formed in 400 μg/gel-, but not 80 μg/gel-treated samples than in contralateral samples at 12 weeks after operation [Fig. 5(D): 80 μg, $P=0.07$; 400 μg, $P=0.009$]. This difference was not observed in samples at 4 weeks [Fig. 5(C)]. These results suggest that the EP2 agonist improved the environment surrounding cartilage.

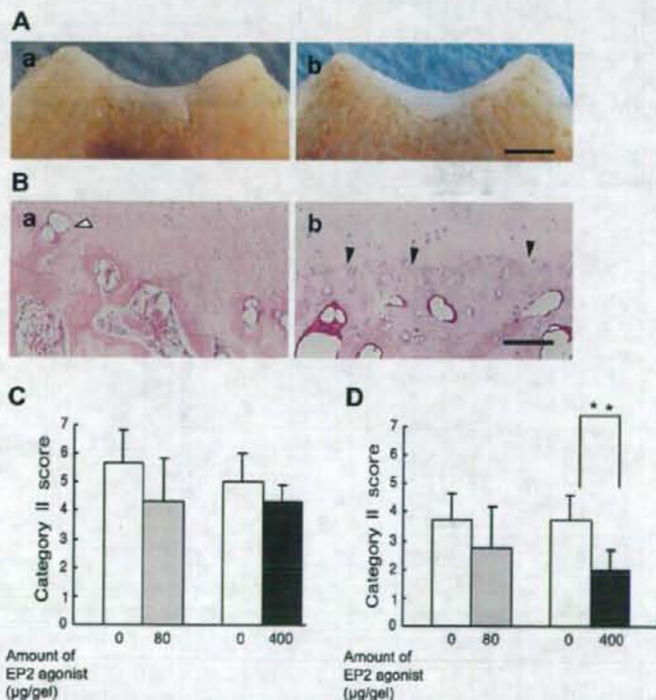

Fig. 5. Effect of EP2 agonist on remodeling of the deep layer zone in osteochondral defect model. A: Macroscopic view of specimens from contralateral sample (a) and EP2 agonist-treated (400 μg/gel) sample (b) of osteochondral defect model at 12 weeks after the operation. Bar = 4.0 mm. B: Microscopic view of the osteochondral boundary in the specimens presented in (A). (a) Contralateral sample (b) EP2 agonist (400 μg/gel)-treated sample. Note that there was no clear boundary, and vascular invasion into articular chondrocytes (white arrowhead) was observed in (a), whereas a clear tidemark (black arrowhead) was formed in (b). Magnification 40×. Bar = 100 μm. C and D: Quantitative evaluation of the osteochondral boundary. The boundary formed between articular cartilage and subchondral bone was evaluated by category II scale of the modified Wakitani scale (0–7, 7 is worse). Samples were prepared from the chondral defect model at 4 weeks (C) and at 12 weeks (D), $^{**}P<0.01$.

EP2 AGONIST DID NOT INDUCE INFLAMMATION OF THE SYNOVIUM

It is important whether the EP2 agonist induced unfavorable inflammation during the regeneration process. We evaluated the synovium based on mRNA expression and histological scoring, and also measured the amounts of cytokines in joint fluid at 4 weeks. PGE2 was reported to up-regulate the expression of the *MMP3*, *TIMP3*, and *IL-1β* genes[3]. The expression of these inflammation-related genes was analyzed by semi-quantitative [Fig. 6(A)] and quantitative RT-PCR [Fig. 6(B)] using samples taken at 4 weeks. No significant up-regulation was found in either gene even in samples treated with a larger amount of EP2 agonist [Fig. 6(B)]. The activity of MMP3 in joint fluid had almost the same value as found in untreated joints [Fig. 6(C)]. The amount of TNF-α or CRP in treated knee joints showed no significant change compared with that in normal joints either [Fig. 6(D)]. Lining synovial cells showed no numerical or morphological change on treatment of with the agonist (400 μg/gel) [Fig. 6(E)], and the histological scoring for inflammation in the treated side was equal to that in the contralateral side [Fig. 6(F)]. These results

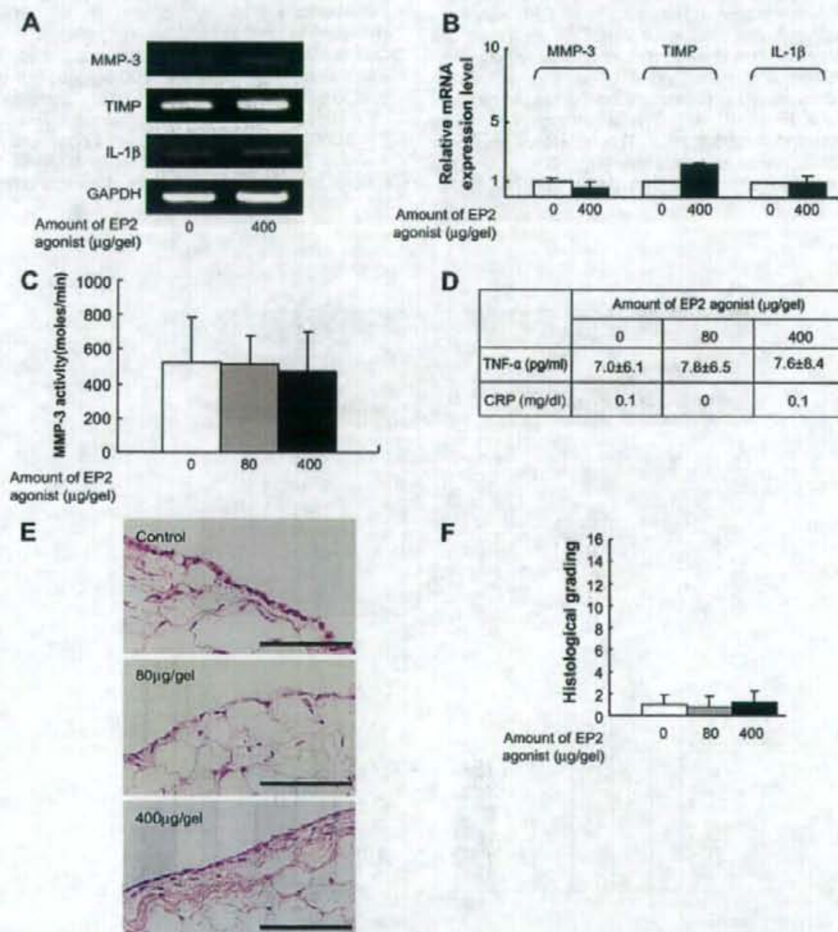

Fig. 6. EP2 agonist did not induce inflammation of the synovium. Inflammatory signs in knee joints were investigated in contralateral or EP2 agonist (80 or 400 μg/gel)-treated samples at 4 weeks after the operation in the osteochondral defect model. A and B: mRNA expression of inflammatory cytokines of cells in the synovium. RNA extracted from synovium was analyzed by semi-quantitative (A) and quantitative (B) RT-PCR. C: Activity of MMP3 in joint fluid. Joint fluid was collected from knee joints of contralateral (open square) and EP2 agonist-treated (blackened square) samples, and the activity of MMP3 was analyzed as described in the Materials and methods section. Five samples were analyzed in each group. D: Amount of TNF-α and CRP in joint fluid. Joint fluid was collected from five knee joints in each group. E: HE staining of synovium in knee joints. Specimens were prepared from a contralateral sample (a), and EP2 agonist-treated samples (b, 80 μg/gel; c, 400 μg/gel). Magnification 200×. Bar = 100 μm. F: Histological scoring of inflammation. Five specimens were analyzed in each of the contralateral and EP2 agonist-treated groups.

indicated that the EP2 agonist induced no significant inflammatory reaction in knee joints at the dose used in this study.

Discussion

PGE2 is a major prostanoid synthesized in response to various stimuli in a variety of cells and exerts local and systemic pleiotropic effects. In general, PGE2 plays a role in maintaining the physiological homeostasis, but in some pathogenic conditions such as inflammation and carcinogenesis, the excess PGE2 induced by factors such as inflammatory cytokines worsens the condition[10]. From this standpoint, PGE2 may be regarded as a pro-inflammatory factor promoting the pathological stage of OA. On the other hand, PGE2 has an anti-inflammatory function. Typical anti-inflammatory actions of PGE2 are demonstrated by the suppressive role of the PGE2 signal *via* EP3 in asthma[20]. As for the PGE2 signal *via* EP2, several reports have analyzed the relationship with inflammation. In human periodontal ligament cells, PGE2 signal *via* EP2/EP4 down-regulated the production of MMP3 and IL-6 stimulated by IL-1[21,22]. An EP4 agonist stimulated the production of MMP9 in macrophages, which was not observed on treatment with an EP2 agonist[23]. The expression of *COX-2* and *MMP9* genes was elevated in macrophages from EP2 null mice[24]. These results suggested that PGE2 acting through EP2 has a minimal role as a pro-inflammatory factor.

Our previous[11] and current study suggested that PGE2 signal *via* EP2 is not only anti-inflammatory but also promotes the regeneration of articular cartilage. The regeneration process of osteochondral defect may mimic the endochondral ossification in fracture healing, and several studies have already shown that the PGE2 signal involved in this process[25–28], among which signals *via* EP2 was particularly important[29]. Cells derived from bone marrow may play a central role in this process. On the other hand, the chondral defect model in this study may mimic the some stage of OA, in which cartilage tissue disappeared and subchondral bone was sclerosed. At this stage, the recruitment of bone marrow-derived cells was minimum, and, therefore, restoration of cartilage tissues was hardly observed. The therapeutic effects of EP2 agonist in this type of model, therefore, may be due to its effects directly on the remaining chondrocytes which are at the resting state in physiological condition as indicated by no PCNA staining [Fig. 3(A)]. The effects of EP2 agonist treatment were more prominent at the later time point (12 weeks) in both chondral and osteochondral defect models, suggesting that the regeneration of cartilage tissue was dependent on the growth of articular chondrocytes with low growth property. We observed that the amount of regenerated tissues was much less at 12 weeks than at 4 weeks after operation in control and contralateral samples, suggesting that regenerated cartilage tissues may lack the proper quality to maintain the structure, and the treatment with EP2 agonist may prevent such degeneration.

One of interesting finding in the current study is that the treatment with EP2 agonist enhanced the reconstruction of boundary between articular cartilage and subchondral bone, which is an important factor to maintain the articular structure. We have no clear explanation for this interesting phenomenon. As mentioned above, EP2 agonists stimulate the growth of both cartilage and bone marrow cells, which may relate to the physiological reconstruction of boundary.

It should be noted that the histological scale of contralateral samples tended to be better than that of control samples (Figs. 1 and 2) and the scales of contralateral samples of 400 μg/gel-treated animals were significantly better than 80 μg/gel-treated animals at 12 weeks [Fig. 2(D), $P = 0.02$], suggesting that there might be an effect from the treated-site through the systemic circulation. Although there were no signs of a general effect of PGE2 such as a reduction in blood pressure (data not shown), continuous release of the EP2 agonist may affect tissue regeneration on the contralateral side.

The ideal regeneration-promoting therapeutics will be small molecules which can be produced in a large amount, promote the regeneration of articular cartilage with a physiological structure, and have no adverse effects in other tissues either locally or systemically. No osteophyte formation was observed in any samples described in this study and also samples observed for a longer period (24 weeks) (data not shown). The results of this study suggested that the EP2 agonist is a promising candidate for such a new drug. Because EP4 is not expressed in normal articular chondrocytes[11], we have been focusing the analysis of EP2 agonist. In the case of osteochondral defect model, however, the combination of EP2/EP4 agonist is a reasonable choice to test considering the fact that the simultaneous activation of EP2 and EP4 cooperatively induced type II collagen mRNA expression[7]. The current experimental model has been used in several prior investigations of various articular repair procedures[30], but may not reflect the pathogenesis of OA (no mechanical factor, no inflammation, no aging factor). Further confirmation of the effect of EP2 agonists in combination with a more effective drug delivery system and experimental OA models in larger animals may lead to a new way to treat OA.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Supplementary material

Supplementary material for this article may be found, in the online version, at doi: 10.1016/j.joca.2008.09.003.

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